RESEARCH

Vol.13, 2019

ISSN 2278–540X
EISSN 2278–5396

DRUG DISCOVERY

Development of olive leaf extract loaded fibroin microparticles by spray drying

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Recently, natural bioactive compounds have been the focus of many health related biotechnology studies. In order to enhance the stability of the natural bioactive compounds appropriate carrier systems are certainly needed to protect them from harsh environmental conditions and also control their release rates. In this study encapsulation of olive leave extract (OLE) with silk fibroin was achieved by using spray drying method to prepare functional microparticles with antioxidant and antimicrobial properties. Release profile of encapsulated olive leave extract was also studied. Spray drying of silk fibroin aqueous solution induced the formation of β-sheet structure in the prepared microparticles. Maltodextrin present in microparticles was used to change the dissolution rate partly while silk fibroin was in the insoluble β-sheet structure. As a result, it was possible to obtain partially soluble microparticles allowing sustained release of encapsulated olive leave extract.

INTRODUCTION

Research on and the application of polyphenols, have recently attracted great interest in the functional foods, nutraceutical and pharmaceutical industries, due to their potential health benefits to humans.[18,19] With the recent studies polyphenols gain more and more interest. Olive fruits and virgin olive oils are known as good polyphenol sources. The o-dihydroxyphenolic glycoside oleuropein is one of the main phenolic compounds identified in olive fruits and leaves.[1]

Oleuropein has significant antioxidant and antimicrobial properties which provide important health benefits.[2] In addition, its derivatives which are responsible for the bitter and pungent taste of olive green fruits and virgin olive oil, also have antioxidant properties.[3] Enzymatic biotransformation of oleuropein in the olive plant is related with the fruit maturation and with tissue-specific defense mechanism which prove the oleuropein-derivatives having antimicrobial activity.[4] Recently antioxidants like oleuropein and rutin gain so much attention due to their high antioxidant capacities. These polyphenols have potentials to be used as remedy for many diseases like cancer, diabetes.[5] However; the effectiveness of polyphenols depends on preserving their stabilities, bioactivities along with enhanced bioavailability. The unpleasant taste of most phenolic compounds also limits their applications.[6]

The main objective of encapsulation is to protect the core material including bioactive compounds from adverse environmental conditions, such as undesirable effects of light, moisture, and oxygen, thereby contributing to an increase in the shelf life of the product, and promoting a controlled release of the encapsulated bioactive compounds.[7]

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With the encapsulation of bioactive compounds, stability and bioavailability problems of the natural compounds can be solved. In addition to that it can improve their functional properties such as antioxidant activities. The production of encapsulated polyphenols, instead of free counterparts, can effectively decrease these undesired drawbacks.[8]

In the literature encapsulation of plant-derived extracts were done by using encapsulation materials such as, chitosan, gelatin, maltodextrin, Arabic gum, starch. In our study silk fibroin together with maltodextrin was used for the encapsulation of olive leave extract. Conventionally silk has been used in the textile industry; however over the years it has been used widely in the biotechnological and biomedical applications.[9] Silk fibroin is a biodegradable and biocompatible natural protein which can be used for the preparation of drug delivery systems. The structure of the silk fibroin consists of amino acids which are beneficial to health as well. Moreover, in the literature it is stated that, polyphenols in the olive leaves like oleuropein and rutin can be adsorbed on the silk fibroin having hydrophobic properties.[10] So, by using silk fibroin for the encapsulation of olive leave extract it is possible to produce a functional food and food supplement without jeopardize the activity of the polyphenols.

Spray dryer method is one of the most preferred encapsulation techniques due to the fact it is economical, easily applicable and flexible. It produces good quality particles. Therefore it is the most widely used microencapsulation technique in the food industry and it is typically used for the preparation of dry, stable food additives and flavors.[11]

In this study olive leave extract with antimicrobial and antioxidant properties was encapsulated into microparticles by using silk fibroin and maltodextrin together. Effects of the parameters of the spray dryer on the morphology of the microparticles were investigated.

MATERIALS AND METHODS

Materials

Olive leaves were collected from the olive trees in the Aegean region of Turkey. Ethanol was purchased from Sigma Aldrich. Dialysis tube was purchased from Thermo Fischer. Oleuropein and Hydroxytyrosol standards were purchased from Extrasynthese. Phosphate buffer tablets were purchased from Sigma Aldrich.

Preparation of olive leave extract (OLE)

The collected olive leaves were washed with deionized water and then dried at 37 °C. After the leaves dried, they were grounded and used for extraction. For the extraction the ratio of plant material (olive leave) to liquid (70% aqueous ethanol solution) ratio was chosen as 1:20. Extraction was carried out at 30 °C for two hours. Ethanol was removed by using rotary evaporator. Remaining olive leave extract aqueous solution was dried by using a freeze dryer to obtain driedolive leaf extract.

Preparation of silk fibroin (SF) solution

Raw silk was kept in boiling water (0.05% Na_2CO_3) for 60 min. This treatment was repeated three times. After degumming (removal of sericin) raw silk was treated with Ajisawa's reagent ($CaCl_2$/ethanol/water, 111/92/144 in weight). The mixture was stirred at 75 °C for two hours. The prepared fibroin solution was dialyzed in a cellulose tube (ThermoFisher SnakeSkin) against deionized water.

Preparation of solutions with silk fibroin, maltodextrin and olive leave extract

Aqueous solutions with silk fibroin, maltodextrin (MD) and olive leaf extract at predetermined concentrations were prepared. In the solutions to be spray dried silk fibroin concentration was kept at 5% (w/v). Concentration of olive leave extract and maltodextrinin solutions was changed between 2.5 and 5 % (w/v). Maltodextrin was used in order to prevent the foaming of silk fibroin in the spraying nozzle and adjust the solubility of prepared microparticles.

Preparation and characterization of silk fibroin microparticles

The prepared solutions were fed to the nozzle oflab scale spray dryer (NPRDU Ltd. Co., İzmir, Turkey) at a constant flow rate of 10 ml/min. Air flow rate was kept constant at 35m^3/h. Spray drying was performed atthree different air inlet temperatures(120, 140, and 160°C).

A Fourier transform infrared (FT-IR) spectroscopy (Perkin Elmer, USA) was used to confirm the structures of the components present in microparticles.The morphology of the prepared microparticles was observed using a scanning electron microscope (JEOL, Japan).

Determination of total phenolic content and antioxidant capacity

The total phenolic content of the extract encapsulated in microparticles was determined by the Folin–Ciocalteu method. The microparticles were dissolved in DMSO (1:20, w/v). After proper dilutions 500 µl sample was brought to a final volume of 3 mL with Folin–Ciocalteu reagent. Then 2 ml of 7.5% (w/v) sodium carbonate was added. The mixture was allowed to stand for 60 min in the dark at room temperature, and absorbance was measured at 725nm. The total phenolic content was calculated from the gallic acid calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Trolox equivalent antioxidant capacity (TEAC) method[12] was used in order to determine the total antioxidant capacity of the microparticles with olive leaf extract.

The antioxidant analysis method was based on the ability of olive leaf antioxidants to scavenge the ABTS radical cation compared with Trolox. Aqueous ABTS solution of 7mM was mixed with 2.45mM potassium peroxodisulfate solution to form ABTS cation followed by incubation in dark at room temperature for 16 h. The ABTS solution was diluted with ethanol until absorbance reach 0.7 (±0.03) at 734 nm prior to use. Antioxidant capacity of the microparticles was given as mM Trolox/g dry weight.

Determination of antimicrobial activity

Antimicrobial activity was determined against *Escherichia coli* (NRRL B-3008) and *Staphylococcus epidermidis* (ATCC 12228)using the modified broth micro-dilution method.[13] Microparticles were dissolved in broth medium to prepare a solution at a constant concentration of 10 mg /ml. The filtered solution was added to wells of a microplate along with microbial suspensions. The microplate was then incubated at 37°C for 16h. Microbial growth was recorded as change of absorbance values at 600 nm in the wells. Inhibition of growth was determined with the observation of unchanged absorbance values with time.

Release of olive leave extract from microsparticles

Release studies of OLE loaded microsparticles were carried out at a batch system with phosphate buffer (pH=7.4) at 37°C and 100 rpm. Releases of OLE from microspheres were investigated by following the changes in total phenol content in release medium with time. Cumulative release percent was graphed as a function of time and release behaviors of the microparticles were compared with each other.

RESULTS AND DISCUSSION

Morphology of microparticles

Scanning Electron Microscope (SEM) images of the microparticles prepared with a spray dryer under different conditions are given in Figures 1 to 8. The sizes and the morphology of the microparticles were investigated with changing parameters like inlet air temperature, and the amounts of OLE and MD in solutions to be spray dried. Effect of MD on the morphology of the microparticles can be seen in Figure 1 and 2. The only difference for the preparation of microparticles observed in Figure 1 and Figure 2 is the presence of MD in the aqueous SF solution containing OLE.

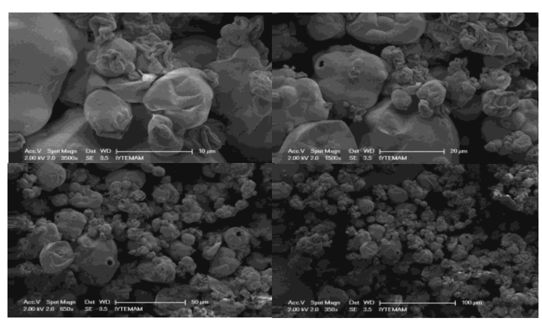

Figure 1 SEM images of SF microparticles including OLE. Spray conditions: solution including OLE 2.5%, SF 5% (w/v), Inlet Air Temperature 140°C.

As seen from Figure 1 the obtained particles have wrinkled surfaces. On the other hand, it can be seen from Figure 2 the obtained particles have smoother surfaces with relatively more uniform size distribution.

With the addition of MD into the mixture, as expected the obtained particles have smoother surfaces due to the fact MD broke the foam in the nozzle and allowed better drying for the particles. So, for the further

experiments MDwas used to have smoother and microparticles close to spherical shapes.

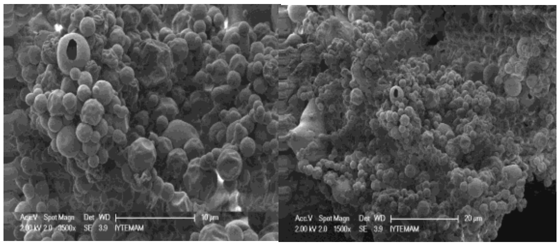

Figure 2 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 2.5%, SF 5%, and MD 2.5% (w/v), Inlet Air Temperature 140°C.

In Figure 3 and 4 the effect of the inlet air temperature on the morphology of microparticles is clearly demonstrated. For these sets the concentration of OLE, MD and SF were kept same at 2.5, 2.5 and 5%, respectively. The inlet air temperature for the preparation of microparticles observed in Figure 3 and 4 are 120, and 160 ºC respectively.

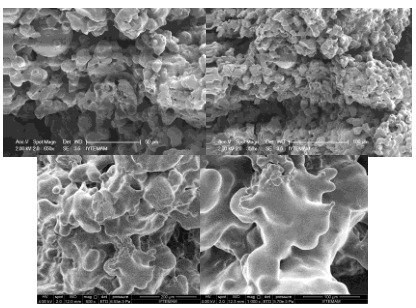

Figure 3 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 2.5%, SF 5%, and MD 2.5% (w/v), Inlet Air Temperature 120°C.

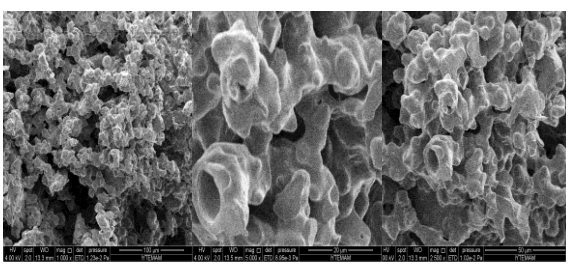

Figure 4 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 2.5%, SF 5%, and MD 2.5% (w/v), Inlet Air Temperature 160°C.

As seen from the Figure 3 120°C was not enough to form properly dried spherical particles. The reason is that 120°C inlet air temperature was not enough to evaporate the all water in the mixture which led to non-uniform irregular structures as observed in Figure 3.

In the figure 4 it can be seen that the obtained particles have wrinkled surfaces due to the rapid evaporation of the water in the mixture. When it is compared with the microparticles observed in Figure 2, 140°C for the inlet air temperature for these conditions is the optimum temperature.

Effect of OLE amount in the solution to be spray dried on the morphology of microparticles can be seen in Figure 5.

As seen from Figure 5, microparticles with the smoothest surfaces were obtained under these conditions. With the increasing OLE amount, solid content of the solution was increased which allowed better drying for the particles.

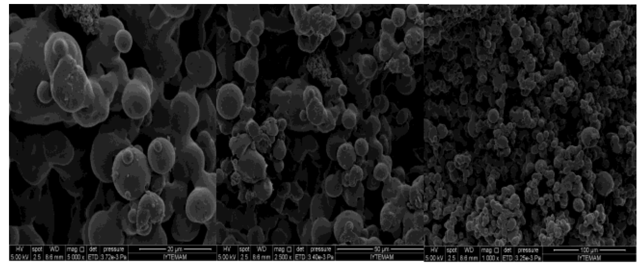

Figure 5 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 5%, SF 5%, and MD 2.5% (w/v), Inlet Air Temperature 140 ° C.

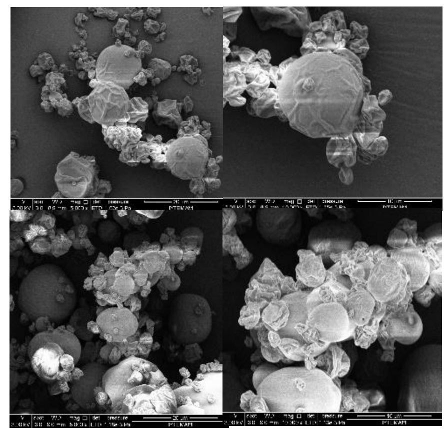

Figure 6 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 5%, SF 5%, and MD 2.5% (w/v), Inlet Air Temperature 160°C.

Microparticles prepared at the inlet air temperature of 160 °C can be seen in Figure 6. Even though the solid content of solution was increased, 160°C was still too high for the drying process. High inlet air temperature caused the formation of microparticles with wrinkled surfaces due to the rapid evaporation.

For the final step the amount of MD was increased to 5% (w/v) in the solution. SEM images of the obtained microparticles were given in Figure 7.

As seen from Figure 7, with the increasing MD amount in the solution better drying conditions were provided. The obtained particles had relatively smooth surfaces and uniform sizes. When all the results are compared the best spray drying can be achieved with a solution having OLE 5%, SF 5% and MD 5% at inlet air temperature of 140°C. For these spray drying conditions the size of the microparticles were in the range of 10-35 µm. Average particle size was determined as 25 µm.

FT-IR Spectra for only SF microparticles, maltodextrin and SF microparticles (33%OLE w/w) prepared from solution having 2.5 % OLE, 5% SF are given in Figure 8A-C, respectively. With the FT-IR analysis it was observed that, spray dried silk fibroin microparticles showed characteristic β-sheet structure. Amide I band shifted from 1650 cm^{-1} to 1632 cm^{-1}. Same shift was observed for Amide II band from 1535 cm^{-1} to 1516 cm^{-1}. In the analyzed samples following peaks were observed 3300 cm^{-1} (N-H), 1650 cm^{-1} (amide I), 1516 cm^{-1} (amide II), 1232 cm^{-1} (amide III). In addition, peaks at 1516 cm^{-1} and 1065 cm-^{1} were observed which also indicated the presence of β-sheet structure in the microparticles. All these results showed that, spray drying of silk fibroin aqueous solution to prepare microparticles induced the formation of β-sheet structure. These results are in accordance with the findings

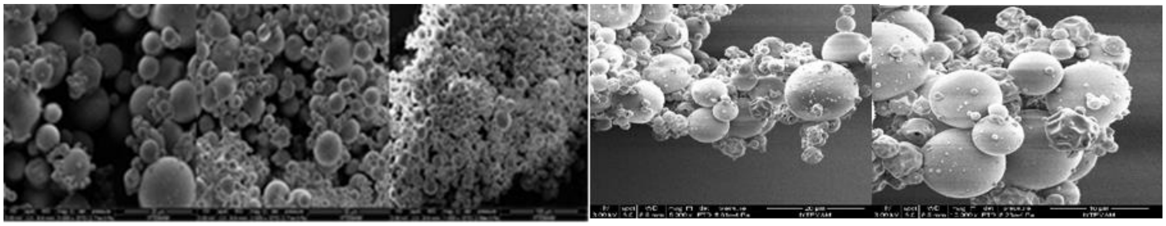

Figure 7 SEM images of SF microparticles including OLE and MD. Spray conditions: solution including OLE 5%, SF 5%, and MD 5% (w/v), Inlet Air Temperature 140 ° C.

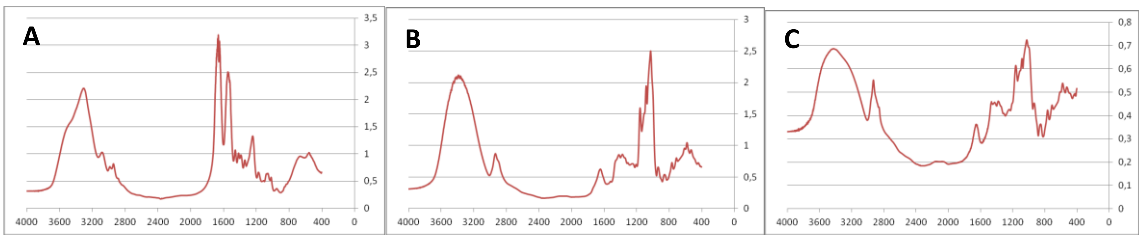

Figure 8 FT-IR spectra for only SF microparticles (A), maltodextrin (B) and SF microparticles (33%OLE w/w) prepared from solution having 2.5 % OLE, 5% SF (C).

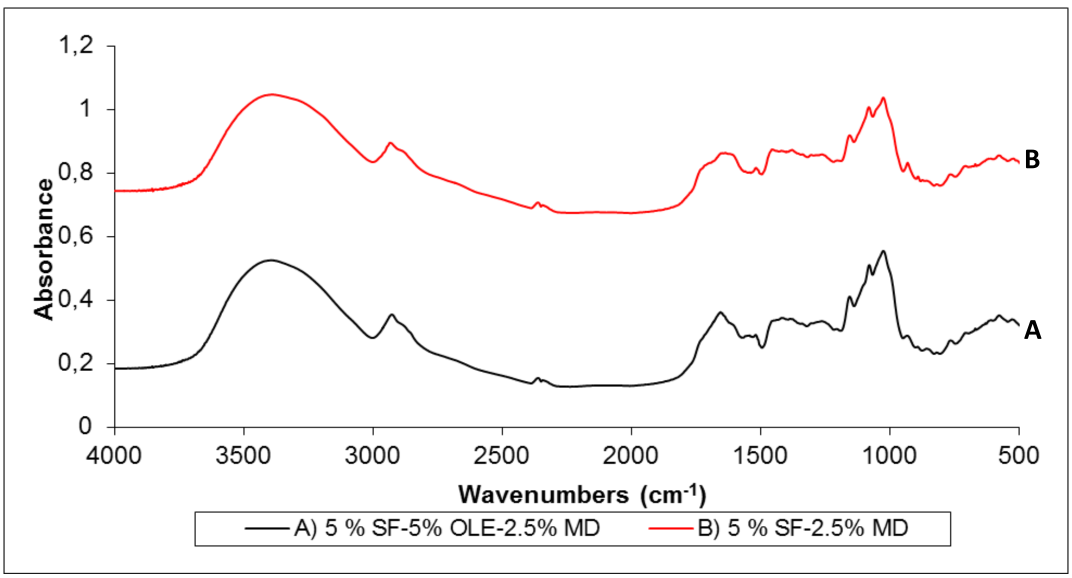

Figure 9 FT-IR spectra for microparticles prepared from A) solution having (5% SF, 5 % OLE, 2.5% MD) and B) solution having (5% SF, 2.5% MD).

reported earlier in the literature [14-16]. The peaks observed in the 1012 and 992 cm^{-1} were characteristic C–O stretching bands for maltodextrin. In the microparticles maltodextrin was used to change the dissolution rate partly while silk fibroin was in the insoluble β-sheet structure. Therefore, it was possible to obtain partially soluble microparticles loaded with olive leave extract. As seen in Figure 9 A-B characteristic peaks for polyphenolic compounds which are natural bioactive compounds in olive leave extract, were observed between 900 and 1200 cm^{-1}. In olive leave extract loaded silk fibroin microparticles intensity of these peaks increased.

Total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC) of the olive leave extract encapsulated in microparticles

Total phenolic content and the total antioxidant capacity of the olive leave extract encapsulated in silk fibroin microparticles spay dried at 140°C were measured and tabulated in Table 1.

Antioxidant and antimicrobial activity of olive leaf extract having oleuropein as major compound are quite well known in the literature.[2, 5, 10]

As seen from Table 1 the fibroin microparticles without olive leave extract did not show any phenolic content and antioxidant capacity as expected. In Table 1 the weight percentage of OLE in microparticles are given in parenthesis. With the increasing amount of olive leave extract in fibroin microparticles phenolic content and antioxidant capacity of the microparticles increased. Maltodextrin in the fibroin particles was used for the purpose of adjusting the solubility and controlling release profile of OLE. The microparticles without maltodextrin had lower solubility due to β-sheet structure of silk fibroin.

Determination of antimicrobial activity of the olive leave extract encapsulated in microparticles

In the literature many studies reported the antimicrobial properties of the olive leaf extract against different pathogen microorganisms including *E.coli, K.pneumoniae, S.aureus, S.epidermidis.*[17] The antimicrobial

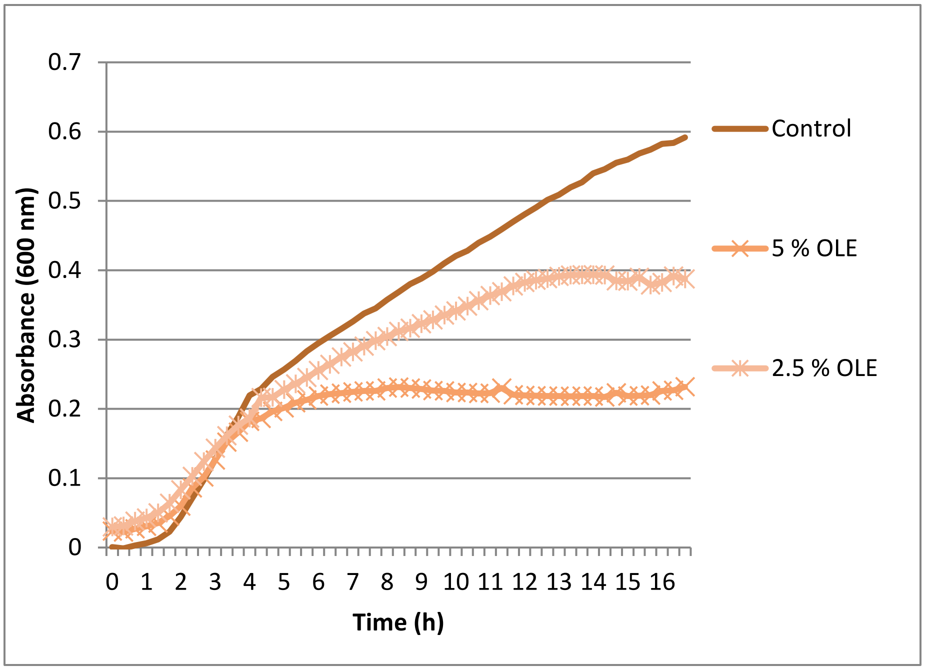

Figure 10 Effect of fibroin microparticles with OLE on the inhibition of growth curve for *E.coli*.

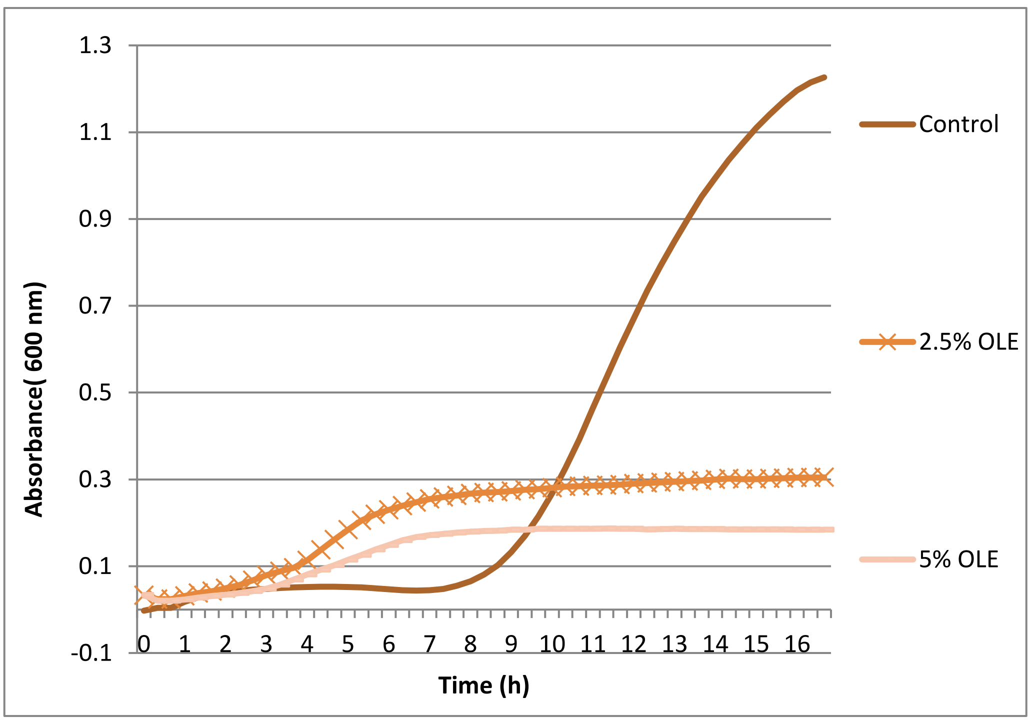

Figure 11 Effect of fibroin microparticles with OLE on the inhibition of growth curve for *S.epidermidis.*

Table 1 Total phenolic content (TPC) and the Trolox equivalent antioxidant capacity (TEAC) of the olive leaf extract encapsulated in silk fibroin microparticles spay dried at 140°C.

OLE % w/v in solution (% w/w in microparticles)	MD % w/v in solution (% w/w in microparticles)	SF% w/v in solution (% w/w in microparticles)	TPC (mg Gallic Acid/mg dry weight)	TEAC (μM Trolox/mg dry weight)
-	-	**5** (100)	-	-
2.5 (33.3)	-	**5** (66.7)	**0.043**	**157.48**
2.5(25)	**2.5** (25)	**5** (50)	**0.030**	**101.45**
5 (40)	**2.5** (20)	**5** (40)	**0.061**	**276.65**
5 (33.3)	**5** (33.3)	**5** (33.3)	**0.038**	**173.97**

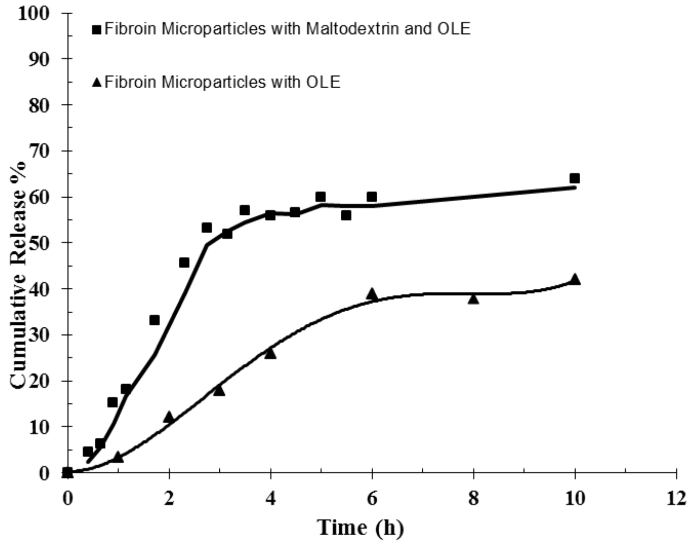

Figure 12 Release profiles of total phenolic contentin olive leave extract encapsulated in fibroin microparticles.

activities of the olive leave extract encapsulated in fibroin microparticles against *E.coli* and *S.epidermidis* are given in Figures 10 and 11, respectively.

The growth curves labeled as control for the *E.coli* and *S.epidermidis* are given in Figures 10 and 119, respectively. The microparticles prepared from solution (2.5% OLE, 2.5%MD, 5%SF; all w/v) contained 25% OLE (w/w). The microparticles prepared from solution (5% OLE, 2.5%MD, 5%SF; all w/v) contained 40% OLE (w/w). Microorganisms were inoculated to the broth solution including solid free extract of these microparticles with 20 and 40 % (w/w) OLE content. Growth of both microorganisms was significantly inhibited indicating the antimicrobial activities of fibroin microparticles including OLE.

Release of olive leave extract from microparticles

The release profiles of olive leave extract from fibroin microparticles are given in Figure 12. Release of the OLE was measured as amount the total phenolic content in the phosphate buffer as release medium.

As seen from Figure 12 total phenolic content released from the fibroin microparticles increased with time. Release of OLE occurred in two stages at different release rates when the release of total phenolic content from microparticles (33.3% OLE w/w) prepared from solution (5% OLE, 5%MD, 5%SF; all w/v) was investigated. For the first 4 hour release rates of OLE was relatively higher due to the high water solubility of maltodextrin in the microparticles. Almost 55% of the total phenol content of the microparticles was released within 4 hours due to the presence of maltodextrin in the particles. Then for the next 6 hours the release slowed down and reached only 65 % of the total phenol content of the microparticles due to the β sheet structure of the silk fibroin in the particles.The β sheet structure of the silk fibroin is insoluble in water and hydrophic in nature causing the slow release due to the significant hydrophobic interaction between fibroin and polyphenols available in OLE. The presence of hydrophobic interactions between silk fibroin and polyphenols were also reported in several studies in the literature.[10, 17]

Sustained release of OLE occurred during 10 hours when the release of total phenolic content from microparticles (33.3% OLE w/w) prepared from solution (2.5% OLE, 0%MD, 5%SF; all w/v) was investigated. Almost 45% of the total phenol content of the microparticles was released within 10 hours in the absence of maltodextrin in the microparticles. Again sustained release of the OLE polyphenols from microparticles made of only silk fibroin can be attributed to presence of the β sheet structure of the silk fibroin.

CONCLUSION

Silk fibroin microparticles containing olive leaf extract (rich in polyphenols) with antioxidant and antimicrobial activity were successfully obtained by using a spray dryer. With the increasing amount of olive leave extract in fibroin microparticles phenolic content and antioxidant capacity of the microparticles increased. Maltodextrin in the fibroin microparticles was successfully used for the purpose of adjusting the solubility and controlling the release profile of OLE from microparticles. Sustained release of the OLE polyphenols from microparticles was attributed to presence of the β sheet structure of the silk fibroin induced by the spray drying conditions.

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Article Keywords:
Olive leaf extract, silk fibroin, microparticles, antimicrobial activity, antioxidant activity

Funding:
This study has not received any external funding.

Conflict of Interest:
The authors declare that there are no conflicts of interests.

Peer-review:
External peer-review was done through double-blind method.

Data and materials availability:
All data associated with this study are present in the paper.

Article History
Received: 20 December 2018
Accepted: 04 February 2019
Published: March 2019

Citation
Oguz Bayraktar, Merve Deniz Köse, Yucel Baspinar. Development of olive leaf extract loaded fibroin microparticles by spray drying. *Drug Discovery*, 2019, 13, 39-45

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